

Exhibit 22

Photochemistry and Photobiology

Volume 73 • No. 2 • February 2001

Published by
The American Society
for Photobiology

Photochemistry and Photobiology

The official Journal of the American Society for Photobiology

Editor

J. C. Scaiano, Ph.D.
Department of Chemistry
University of Ottawa
10 Marie Curie, Rm. 306
Ottawa, ON K1N 6N5 Canada
Phone: 613-562-5634
Fax: 613-562-5633
e-mail: P&P@photo.chem.uottawa.ca

Associate Editors

Jean Cadet, *CEA/Département de Recherche
Fondamentale sur la Matière Condensée,
Grenoble, France*

Frank R. de Gruijl, *Utrecht University
The Netherlands*

Xing Wang Deng, *Yale University
New Haven, CT*

Thomas Foster, *University of Rochester,
Rochester, NY*

Barbara W. Henderson, *Roswell Park
Cancer Institute, Buffalo, NY*

Margaret L. Kripke, *Anderson Cancer
Center, Houston, TX*

Francesco Lenci, *Consiglio Nazionale delle
Ricerche, Italy*

Miguel Angel Miranda, *Universidad
Politecnica de Valencia, Valencia, Spain*

Thomas A. Moore, *Arizona State
University, Tempe, AZ*

Hasan Mukhtar, *Case Western Reserve
University, Cleveland, OH*

Harald Paulsen, *Johannes-Gutenberg-
Universität, Mainz, Germany*

Robert W. Redmond, *Harvard Medical
School, Boston, MA*

Aziz Sancar, *The University of North
Carolina at Chapel Hill, Chapel Hill, NC*

John L. Spudich, *University of Texas
Medical School, Houston, TX*

Manfred Tevini, *Universität Karlsruhe,
Karlsruhe, Germany*

Dennis P. Valenzano, *University of Kansas
Medical Center, Kansas City, KS
(electronic publication)*

Photochemistry and Photobiology (ISSN 0031-8655) is published monthly, two volumes per annum, by the American Society for Photobiology, BioTech Park, 1021 15th Street, Suite 9, Augusta, GA 30901-3158, USA.

Editorial Advisory Board: R. K. Crouch, Charleston, South Carolina; P. L. Dutton, Philadelphia, PA; J. E. Hearst, Berkeley, CA; I. E. Kochevar, Boston, MA; L. H. Pratt, Athens, GA; R. B. Setlow, Upton, NY

Subscriptions: The 2000 Annual Institutional Subscription Rate is \$590 for 12 monthly issues plus Meeting Abstract issue. Institutional subscription information is available from Allen Press, Inc., 1041 New Hampshire Street, P.O. Box 368, Lawrence, KS 66044; Phone 785-843-1221, Fax 785-843-1274. Individual members of the American Society for Photobiology receive a complimentary subscription. Membership information may be obtained from the Society office at the address listed above or at <http://www.POL-US.net>.

Instructions to Authors: Condensed instructions appear at the end of each issue. Complete instructions for preparation of manuscripts and figures and for submission of manuscripts on disk appear in the first issue of each year and are available from the Editorial Office: *Photochemistry and Photobiology*, Department of Chemistry, University of Ottawa, 10 Marie Curie, Rm. 306, Ottawa, ON K1N 6N5 Canada. Phone: 613-562-5634, Fax: 613-562-5633, e-mail: P&P@photo.chem.uottawa.ca. Complete information is also available on the *Photochemistry and Photobiology* home page.

Internet address: The address for the *Photochemistry and Photobiology* home page is <http://www.POL-US.net>. The home page provides information on published papers and manuscripts prior to publication. It also includes information on subscriptions, the aims and scope of the Journal, preparation of manuscripts, and advertisements.

Advertisements: Advertising rates and information are available from the Editorial Office at the address listed above and on the home page.

Change of address: Institutional subscribers should forward their new address to Allen Press at the address for subscriptions given above. Members of the American Society for Photobiology should send new addresses to the Society office listed above.

Back issues: Claims for undelivered issues must be received within six months of the mailing date. Single issues and complete volumes can be purchased depending on availability. Contact Allen Press at the address for subscriptions given above.

Indexing/Abstracting Services: The Journal is currently included by the following services in print and/or electronic format: Current Contents (Life Sciences, Science Citation Index, SCISEARCH Data, ISI/BIOMED Database, ASCA), Chemical Abstracts Service, Index Medicus, MED-LINE, BIOSIS Data, Cambridge Science Abstracts, CABS, and PASCAL-CNRS Data.

Copyright information: *Photochemistry and Photobiology* is copyrighted by the American Society for Photobiology. No portion(s) of this journal may be reproduced without the written consent from the copyright holder. Permission to reproduce copies of articles for non-commercial use may be obtained from the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923, 978-750-8400, for a fee of \$5.00 per copy.

Periodicals postage paid at Augusta, GA and additional mailing offices.

Postmaster: Send address corrections to *Photochemistry and Photobiology*, American Society for Photobiology, 1021 15th Street, Suite 9, Augusta, GA 30901-3158.

Founding Editors: E. J. Bowen, Oxford, UK; S. Claesson, Uppsala, Sweden; A. Hollaender, Oak Ridge, TN; D. Shugar, Warsaw, Poland; A. D. McLaren, Berkeley, CA (Editor, 1962-1965).

Past Editors: K. C. Smith, Stanford, CA (1966-1971); J. Jagger, Dallas, TX (1972-1974); P.-S. Song, Lincoln, NE (1975-1993); Irene E. Kochevar, Boston, MA (1994-1997).

Editorial Associate: Anita Cheslock

Editorial Assistants: Cheryl Cole, Adriana Laliberté, Maria Pilar Fernandez Lorente and Betty Yakimenko

Copyright © 2001 American Society for Photobiology

The paper used in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of paper).

5-Aminolaevulinic Acid Methyl Ester Transport on Amino Acid Carriers in a Human Colon Adenocarcinoma Cell Line[†]

Odrun A. Gederaas^{1,2}, Andrew Holroyd³, Stanley B. Brown³, David Vernon³, Johan Moan² and Kristian Berg^{*2}

¹Institute of Laboratory Medicine, Department of Clinical Chemistry, Medical Faculty, Norwegian University of Science and Technology, Trondheim, Norway;

²Department of Biophysics, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway and

³Center for Photobiology and Photodynamic Therapy, School of Biochemistry and Molecular Biology, University of Leeds, Leeds, UK

Received 6 September 2000; accepted 3 November 2000

ABSTRACT

The transport mechanisms of 5-aminolevulinic acid methyl ester (5-ALA-ME) have been studied in a human adenocarcinoma cell line (WiDr) by means of ¹⁴[C]-labeled 5-ALA-ME. The transport was found to be partly Na⁺ dependent, while the extracellular Cl⁻ concentration did not affect the uptake. The transport of 5-ALA-ME into WiDr cells was dependent on the incubation temperature and was found to be completely blocked by the inhibitors of energy metabolism, 2-deoxyglucose and sodium azide. WiDr cells were treated with 10 mM of 14 different amino acids and the substrate specificity of the 5-ALA-ME transporter(s) was analyzed by treating the cells with 23 μM or 1 mM ¹⁴[C]-labeled 5-ALA-ME. The transport of 5-ALA-ME was found to be inhibited to the highest extent, *i.e.* about 60%, by the nonpolar amino acids L-alanine, L-methionine, L-tryptophan and glycine. The uptake of 5-ALA-ME followed an exponential decay with increasing concentration of glycine, reaching a maximum inhibition of uptake of 5-ALA-ME of 55%. Sarcosine, a specific inhibitor of system Gly, did not significantly inhibit 5-ALA-ME transport. In contrast to transport of 5-ALA, 5-ALA-ME does not seem to be taken up by system BETA transporters. In conclusion, the cellular uptake of 5-ALA-ME into WiDr cells seems to be due to active transport mechanisms, involving transporters of nonpolar amino acids.

INTRODUCTION

The use of 5-aminolevulinic acid (5-ALA)[†] has shown great promise for treatment of a number of superficial conditions

but, the use of esterified derivatives is still very much under investigation (1). The treatment induces accumulation of porphyrins, primarily protoporphyrin IX (PpIX), although other more hydrophilic photosensitizers may also accumulate (2–5). These photosensitizers exert a cytotoxic reaction upon exposure to light, mainly mediated *via* formation of singlet oxygen. A major drawback of the treatment with 5-ALA is its poor ability to diffuse through biological membranes because of its low lipophilicity (6). Only superficially located lesions can be treated as a result of the poor penetration of 5-ALA into tissues. A possible solution for this problem may be to use lipophilic prodrugs of 5-ALA. Several chemical modifications have been made, both on the amino- and carboxyl groups, and the resulting compounds investigated for their ability to induce PpIX formation and sensitivity to photoinactivation (2,3,7,8). So far, the most promising derivatives for clinical utilization have been 5-ALA esterified with aliphatic alcohols. Esterification of 5-ALA with long chain (C₆–C₈) alcohols was found to reduce the amount of 5-ALA (as ester) needed to reach the required level of PpIX accumulation by 30–150-fold as compared with nonesterified 5-ALA (2). Although 5-ALA methyl ester (5-ALA-ME) induces PpIX formation less efficiently than 5-ALA, animal experiments, as well as clinical studies, indicate deeper penetration of 5-ALA-ME as compared to 5-ALA upon topical application and with higher specificity for neoplastically transformed skin lesions (9–12).

The mechanisms associated with the uptake of 5-ALA have been studied in a human adenocarcinoma cell line (WiDr) (13,14), but similar uptake studies with 5-ALA-ME have never previously been performed. In this study the mechanisms of the uptake of 5-ALA-ME in WiDr cells have been investigated. The results show that transport mechanisms for 5-ALA-ME are different from that of 5-ALA.

MATERIALS AND METHODS

Cell cultivation. Cells of an established line (WiDr), derived from a human primary adenocarcinoma of the rectosigmoid colon (15),

[†]Posted on the website on 30 November 2000.

*To whom correspondence should be addressed at: Department of Biophysics, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway. Fax: 47-22-93-42-70; e-mail: kristian.berg@labmed.uio.no

[†]Abbreviations: 5-ALA, 5-aminolaevulinic acid; 5-ALA-ME, 5-aminolaevulinic acid methyl ester; GABA, γ-aminobutyric acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; meth-

© 2001 American Society for Photobiology 0031-8655/01 \$5.00+0.00

yl-AIB, α-methylaminoisobutyric acid; PBS, Dulbecco's phosphate-buffered saline; PDT, photodynamic therapy; PpIX, protoporphyrin, IX.

were subcultured in Roswell Park Memorial Institute 1640 medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. The cells were subcultured approximately twice a week and maintained at 37°C and 5% CO₂ in a humid environment.

Chemicals. [4-¹⁴C] 5-ALA (specific activity 47.6 mCi/mmol) was purchased from NEN[®] Life Science Products (Boston, MA). Unlabeled 5-ALA (Sigma, St. Louis, MO, or Photocure, Norway) was dissolved in Dulbecco's phosphate-buffered saline (PBS) or a buffer containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES), 150 mM NaCl, 1.2 mM CaCl₂, 0.64 mM MgCl₂, 6.0 mM KOH and 5.0 mM D-glucose (named HEPES-buffer). pH was adjusted to 7.4 with 5 M NaOH. [¹⁴C] 5-ALA was diluted 48-fold with these solutions containing unlabeled 5-ALA so that the total concentration of 5-ALA was 1 mM. [2,3-³H(N)] γ-aminobutyric acid (GABA) was obtained from NEN[®] Life Science, [1-¹⁴C] β-alanine was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO), while [4-¹⁴C] 5-ALA-ME (specific activity 1.05 mCi/mmol) was synthesized by treatment of [4-¹⁴C] 5-ALA hydrochloride with thionyl chloride in methanol as described below. All other chemicals were of analytical grade and of highest purity commercially available. All amino acids used were L-stereoisomers.

Preparation of ¹⁴C-labeled ALA esters. Forty milligrams of ALA was weighed into a stoppered test tube and 250 µCi ¹⁴C-labeled ALA added (2.5 mL of solution in 0.1 M HCl). This was freeze dried overnight. The freeze dried ALA was dissolved in 1 mL dry methanol (dried with anhydrous sodium sulfate). One milliliter of dry methanol was chilled on dry ice and 0.25 mL thionyl chloride added slowly. The two methanol solutions were mixed and the volume reduced to 1 mL under a jet of nitrogen with gentle warming. About 10 mL of chilled diethyl ether (dry ice) was added with good mixing to precipitate the ester. The tube was stood in dry ice for 5 min. The ester was collected by centrifugation (~1000 rpm for 10 min). This pellets the bulk of the product but the tube warms up during the spin. To ensure all the product was collected the tube was chilled again for 5 min on dry ice and recentrifuged for 5 min. The ether was removed with a Pasteur pipette and discarded. The last of the ether was evaporated under a jet of nitrogen and the product dried thoroughly under vacuum. Thin layer chromatography of all the ester reactions was done using 60:40 acetone:methanol, and visualized by spraying with 2% ninhydrin in acetone and heating in an oven. These analyses showed a radiochemical purity >90%.

Uptake measurements. The cells (4.0–8.0 × 10⁴ cells cm⁻²) were seeded into 24-well plates 2–3 days before the experiments; nearly confluent layers of cells were incubated with radiolabeled material at the desired concentration under various conditions. The cells were either incubated in PBS or in HEPES-buffer. After treatment, the cells were kept on ice and washed four times in cold PBS. The cells were dissolved in 200 µL 0.1 M NaOH. After 10 min of incubation 2 mL scintillation fluid (Opti-fluor, Packard) was added to the samples, and radioactivity was measured in a Packard Tri-Carb 4550 scintillation counter.

Protein determination. Protein was assayed by the Bradford's method (16) using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany) with human serum albumin as standard.

Statistical analysis. Uptake measurements were routinely done in duplicate. Each experiment was repeated two or three times. The results are presented as mean values ± SEM. Linear regression analysis was performed by the method of least squares.

RESULTS

Cellular uptake of 5-ALA-ME

The radioactively labeled compounds [¹⁴C] 5-ALA and [¹⁴C] 5-ALA-ME were utilized for measuring cellular uptake in WiDr cells. The uptake of both 5-ALA and 5-ALA-ME was linear during the 6 h of incubation at 37°C (Fig. 1A). Based on the slopes in Fig. 1A the rate of 5-ALA uptake was 3.6 nmol/mg protein h which is in accordance with previous results (14), while the rate of 5-ALA-ME uptake was about 30% lower, i.e. 2.4 nmol/mg protein h, which is significantly lower than the rate of 5-ALA uptake ($P < 0.05$). The initial

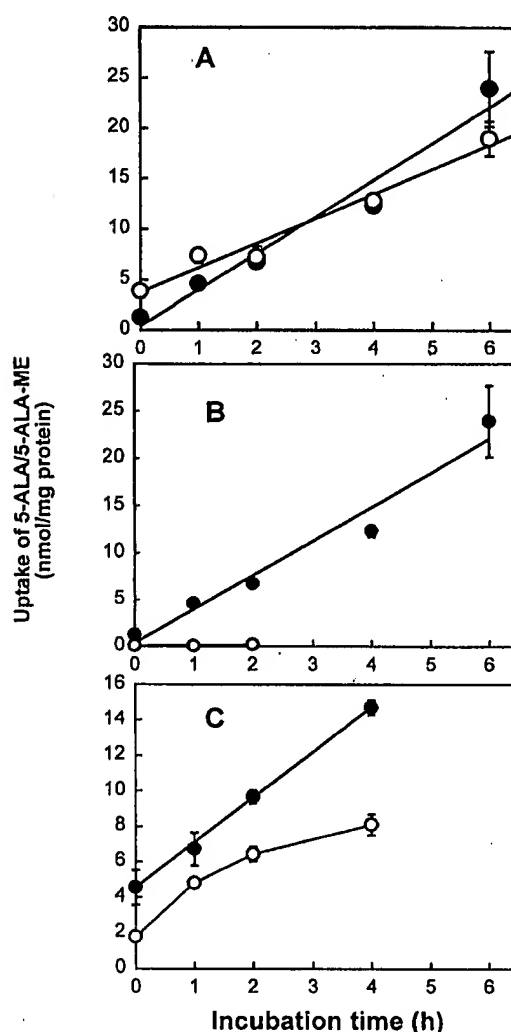


Figure 1. Uptake of 1 mM 5-ALA and 5-ALA-ME in WiDr cells at 0°C and 37°C. (A) Uptake of 5-ALA (●) and 5-ALA-ME (○) at 37°C was measured in HEPES-buffer over an incubation period of 0–6 h as described in "Materials and Methods." The uptake of: (B) 5-ALA; and (C) 5-ALA-ME at 0°C (○) and 37°C (●). Linear regression analysis was performed on the data.

association of 5-ALA-ME with the WiDr cells was higher than that of 5-ALA.

Figure 1B shows the time course of 5-ALA uptake at 0°C and 37°C. For the incubation at 0°C the cells were incubated on ice in a room kept at 4°C. The uptake of 5-ALA at 0°C was found negligible as compared to that at 37°C. In contrast, the uptake rate of 5-ALA-ME at 0°C was initially similar to that at 37°C, but in all the experiments was 55–65% lower 2–4 h after initiation of the incubation (Fig. 1C).

Inhibition of 5-ALA-ME uptake with metabolic inhibitors

To assess the energy dependence of 5-ALA-ME uptake, the 5-ALA-ME uptake was measured in the presence of metabolic inhibitors. The cells were incubated with NaN₃ (10 mM) and 2-deoxy-D-glucose (100 mM), inhibiting the respiration chain and glycolysis, respectively, for 1 h prior to treatment with 5-ALA-ME, resulting in more than 97% re-

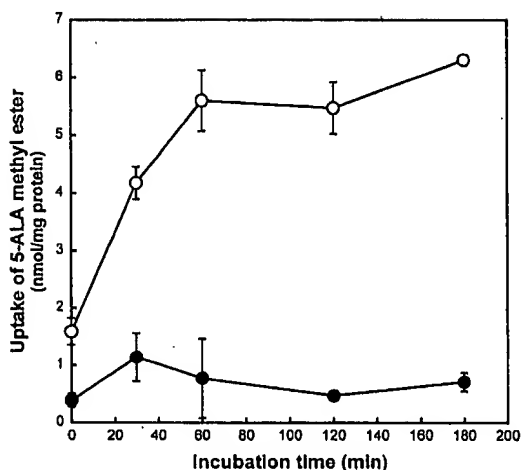


Figure 2. Effects of metabolic inhibitors on the uptake of 5-ALA-ME in WiDr cells. Uptake of 1 mM 5-ALA-ME with (●) and without (○) the presence of the metabolic inhibitors NaN_3 (10 mM) and 2-deoxy-D-glucose (100 mM) was measured as described in "Materials and Methods." The cells were preincubated with metabolic inhibitors for 1 h before 5-ALA-ME was added.

duction in cellular adenosine triphosphate (data not shown). Upon further incubation with the metabolic inhibitors the uptake of 5-ALA-ME was completely inhibited (Fig. 2). It should be noted that the initial association of 5-ALA-ME with the cells in the presence of metabolic inhibitors is about a factor of four lower than the initial binding in the absence of the metabolic inhibitors. The plasma membrane was still intact at the end of the treatment as revealed by the propidium iodine exclusion method (17) (data not shown).

Na^+ dependence of 5-ALA-ME uptake

The Na^+ dependence of 5-ALA-ME uptake in WiDr cells was analyzed by substituting NaCl with choline chloride (Fig. 3). The cells were treated with various concentrations of choline chloride for 3 h, which has previously been found to be nontoxic to the cells (14). As seen in Fig. 3A the uptake of 5-ALA-ME in the cells is partly Na^+ dependent since the uptake is reduced by 40% in the absence of Na^+ ($P < 0.01$ between the groups $[\text{Na}^+] = 0$ and 150 mM). A similar Na^+ dependency was reflected in the 5-ALA-ME-induced PpIX formation (Fig. 3B).

Cl^- dependence of 5-ALA-ME uptake

Chloride ions (Cl^-) were substituted with a mixture of gluconate, NO_3^- and SO_3^- salts (18), and the results show that the 5-ALA-ME uptake is Cl^- independent (Fig. 4). It should be noted that 5-ALA-ME is delivered as 5-ALA-ME HCl, and when all Cl^- is removed from the medium there will be about 1 mM Cl^- left. In contrast to the Cl^- independency of the cellular uptake of 5-ALA-ME extracellular Cl^- concentrations below 90 mM reduced the 5-ALA-ME-induced formation of PpIX (Fig. 4B).

Inhibition of 5-ALA-ME uptake with α -amino acids

WiDr cells were incubated for 3 h with various amino acids (10 mM) and 23 μM or 1 mM 5-ALA-ME containing [^{14}C] 5-ALA-ME (Fig. 5). It was found that nonlabeled 5-ALA-

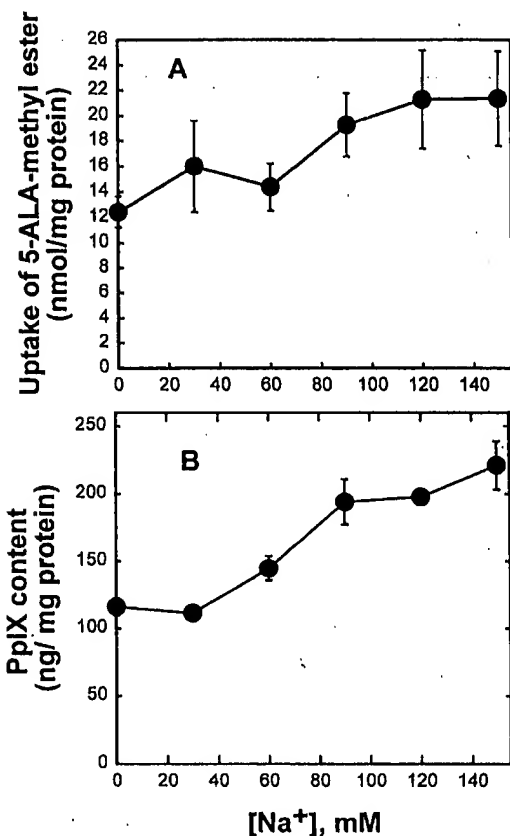


Figure 3. Dependence of: (A) 5-ALA-ME uptake on the Na^+ concentration; and (B) PpIX formation upon incubation of WiDr cells with 1.85 mM 5-ALA-ME and different concentrations of Na^+ . WiDr cells were incubated with 1 mM 5-ALA-ME in a HEPES-buffer in which the Na^+ concentration was varied by substituting NaCl with choline chloride, and otherwise treated as described in "Materials and Methods." Mean \pm SE of three experiments.

and 5-ALA-ME reduced the uptake of [^{14}C] 5-ALA-ME by as much as 41 and 53%, respectively, at low concentrations (23 μM) of the labeled compound, but no significant inhibition was observed at higher concentration of [^{14}C] 5-ALA-ME (1 mM, data not shown). The transport of 5-ALA-ME was inhibited to the highest extent by amino acids with non-polar side chains. It has recently been shown that 5-ALA is transported by system BETA transporters in WiDr cells (14) as well as in *Saccharomyces cerevisiae* (19). A similar inhibitory effect of system BETA substrates on the uptake of 5-ALA-ME was not observed. The inhibitory effect of amino acids on the uptake of [^{14}C] 5-ALA-ME was similar but less pronounced when using the higher concentration of the labeled compound (data not shown).

Inhibition of 5-ALA-ME uptake by sarcosine and glycine

Glycine was one of the most efficient amino acids in inhibiting cellular uptake of 5-ALA-ME (Fig. 5). In cells incubated with 23 μM [^{14}C] 5-ALA-ME the inhibition followed an exponential decay of the form $y = 225 + 285e^{-0.322x}$ (or $y = 0.44 + 0.56e^{-0.322x}$ when noninhibited uptake is normalized to 100) ($R^2 = 0.93$), indicating that a maximum inhibition of 55% of the 5-ALA-ME uptake could be pro-

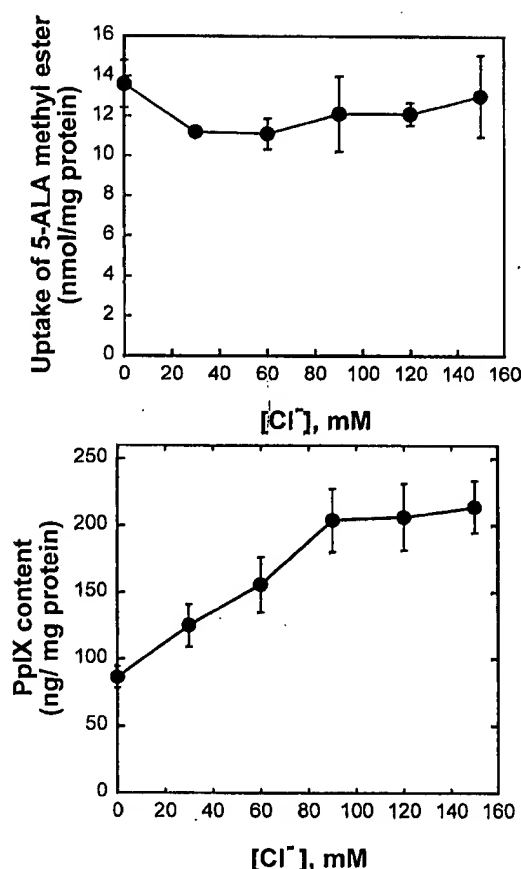


Figure 4. Dependence of: (A) 5-ALA methyl ester uptake on Cl^- concentration (incubation time, 3 h); and (B) PpIX formation on extracellular Cl^- concentration upon incubation of WiDr cells with 1.85 mM 5-ALA-ME. WiDr cells were incubated with 1 mM 5-ALA-ME in a HEPES-buffer in which the Cl^- concentration was varied by substituting NaCl with Na gluconate, $MgCl_2$ with $MgSO_4$ and $CaCl_2$ with $Ca(NaO_3)_2 \cdot 4 H_2O$, and otherwise treated as described in "Materials and Methods." Mean \pm SE of three experiments.

duced by glycine. Similar results with respect to maximum inhibition were obtained when the cells were treated with 1 mM [^{14}C] 5-ALA-ME (data not shown). About 2.5 mM glycine was needed to induce half the maximum inhibitory effect on uptake of 23 μM 5-ALA-ME.

System Gly, a transport system for glycine, is present in several tissues (20). Sarcosine is a specific inhibitor of system Gly and was therefore investigated for its ability to inhibit 5-ALA-ME (Fig. 5). However, the inhibition of 5-ALA-ME uptake by sarcosine was marginal and not statistically significant.

A substantial cellular uptake of 5-ALA-ME was observed at 4°C (Fig. 1). However, the cellular uptake of 1 mM 5-ALA-ME at 4°C was not inhibited by 10 mM glycine (data not shown).

DISCUSSION

Preclinical as well as clinical studies indicate that esterification of 5-ALA with aliphatic alcohols increases the efficiency and specificity of this compound with respect to PpIX formation in target tissues (9–12). In cells in culture the pathways for PpIX formation from 5-ALA and 5-ALA esters

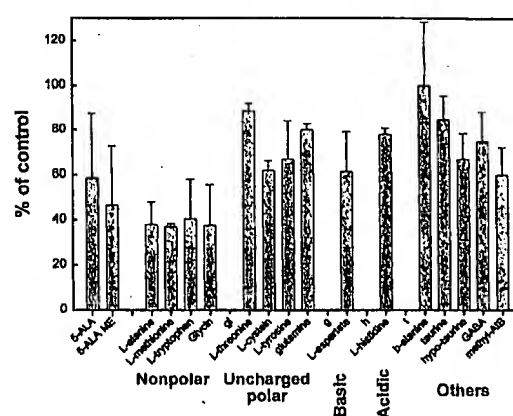


Figure 5. Inhibition of 5-ALA-ME uptake by various amino acids. The amount of 5- $[^{14}C]$ ALA-ME (23 μM) taken up after treatment of WiDr cells for 3 h in the presence of various amino acids (10 mM) is reported as the percentage of the mean uptake of radiolabeled 5-ALA-ME in the absence of any added nonradioactive amino acid. SD for the control, $\pm 11\%$.

diverge only in the first two steps, *i.e.* the transport over the plasma membrane and the deesterification of the ester groups. It has recently been shown that 5-ALA esterified to long chain ($>C_4$) aliphatic alcohols induces formation of PpIX more efficiently than 5-ALA itself, while 5-ALA-ME induces PpIX less efficiently (2,3). In order to reveal the causes for the differences in rates of PpIX formation induced by 5-ALA and its ester derivatives the transport mechanisms for 5-ALA (14) and 5-ALA-ME (this study) have been investigated in an adenocarcinoma cell line.

The present study indicates that in several aspects the transport of 5-ALA-ME is different from that of 5-ALA: the uptake of 5-ALA-ME is less temperature dependent, less Na^+ dependent and, in contrast to uptake of 5-ALA, independent of Cl^- . The inhibition of 5-ALA-ME uptake by amino acids was found to be similar to that for 5-ALA in many cases with the exceptions of methionine, the system BETA substrates and α -methylaminoisobutyric acid (methyl-AIB) (Fig. 5).

The uptake rate of 5-ALA-ME at 0°C and the lack of inhibitory effect of glycine at 0°C might indicate partial uptake of this compound by passive diffusion (Fig. 1C). However, this is contraindicated by the complete inhibition of 5-ALA-ME uptake in the presence of metabolic inhibitors (Fig. 2). The uptake rate of 5-ALA-ME at 0°C is only initially high and is significantly lower after 2 h of incubation, indicating a two-step process with the equilibrium binding of 5-ALA-ME to the plasma membrane reached only slowly at 0°C. It should be noted that the initial association of 5-ALA-ME with the cells at 37°C is much higher than that of 5-ALA (Fig. 1A). In contrast to the zwitterion 5-ALA, 5-ALA-ME is a cation which may be easily associated with the high negative charge on the surface of cells with a high membrane potential. This is consistent with the reduced initial association of 5-ALA-ME with the cells when the plasma membrane potential was lowered (Fig. 2). Thus, the metabolic inhibitors may influence on the cellular uptake rate of 5-ALA-ME by reducing its association with the plasma membrane.

At the same concentration (1 mM) 5-ALA-ME induces

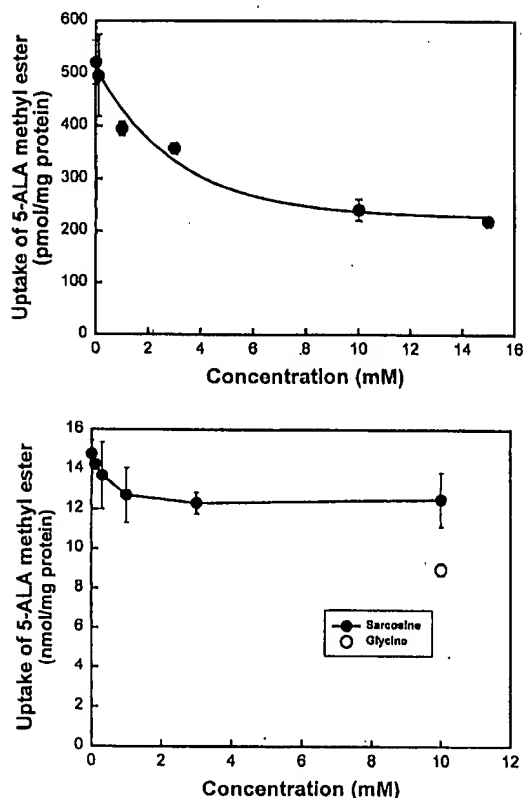


Figure 6. (A) Inhibition of 5-ALA-ME uptake by glycine. The average amount of [14 C] 5-ALA-ME (23 μ M) taken up by WiDr cells after 3 h in the presence of glycine. The curve is the best fit of the results to an exponential decay formula as described in the text. (B) The cellular uptake of 5-ALA-ME in the presence of sarcosine and glycine as indicated on the figure. The cells were incubated with 1 mM 5-ALA-ME containing [14 C] 5-ALA-ME. The cells were incubated in a HEPES-buffer as described in "Materials and Methods."

PpIX formation at a rate more than 60% lower than in the case of 5-ALA (14). This could at least partly be due to a lower rate of uptake of 5-ALA-ME through the plasma membrane (Fig. 1A). This interpretation is further strengthened by the observation of Kloeke *et al.* (21) that fluorescence formation induced by 5-ALA and 5-ALA-ME was similar in lysed T-lymphoma cells, indicating that deesterification of 5-ALA-ME is not a rate limiting step in the PpIX formation.

The present study shows that about 40% of the uptake of 5-ALA-ME is Na^+ dependent (Fig. 3), 50–60% is capable of being inhibited by amino acids (Figs. 5 and 6), the uptake is inhibited about 50% by incubation at 0°C (Fig. 1C) and the uptake at 0°C is not capable of being inhibited by glycine. The inhibition of uptake at 37°C by glycine could be described by an exponential decay function and a maximum of 55% of the 5-ALA-ME transport occurs by carriers of glycine. Altogether, these results indicate that at least two different uptake mechanisms are involved in the cellular uptake of 5-ALA-ME.

The amino acids transporters involved in the uptake of 5-ALA-ME cannot be determined from this study. The cellular uptake of 5-ALA-ME is most efficiently inhibited by the nonpolar amino acids L-alanine, L-methionine, L-tryptophan and glycine (Fig. 5). System Gly, a transporter for glycine, is probably not involved in the uptake of 5-ALA-ME since

sarcosine, a specific inhibitor of system Gly (20), does not inhibit the uptake of 5-ALA-ME and since uptake of 5-ALA-ME was Cl^- independent (Fig. 4) (22). Neither is it likely that transport of 5-ALA-ME by system BETA is of great importance since system BETA substrates, such as β -alanine, taurine, hypo-taurine and GABA, do not inhibit 5-ALA-ME uptake (Fig. 5) (or inhibit to only a minor extent) and since system BETA transporters are Cl^- dependent. This is in contrast to our findings for 5-ALA (14). The two most common Na^+ -dependent transporters are systems A and ASC (20). Transport through system A is inhibited by methyl-AIB (23) which was found to inhibit uptake of 5-ALA-ME by nearly 40% (Fig. 5). Alanine, serine and glutamine are particularly good substrates for system A. Alanine was also found to inhibit transport of 5-ALA-ME in accordance with a role for system A in the transport of 5-ALA-ME. In contrast, glutamine did not significantly inhibit the transport of 5-ALA-ME. System ASC overlaps in specificity with system A, but is not inhibited by methyl-AIB, and alanine, serine and cysteine are preferred substrates (20,24). In accordance with this alanine and to some extent cysteine inhibit uptake of 5-ALA-ME. System B⁰ is another Na^+ -dependent transporter with broad specificity for zwitterionic amino acids, including alanine, glutamine, leucine and phenylalanine (25), of which the specificity for glutamine is not in accordance with transport of 5-ALA-ME. In addition, systems IMINO, X_{AG}⁻ and PHE of the Na^+ -dependent transporters should be considered for the transport of 5-ALA-ME (20). Of the Na^+ -independent transporters system L is one of the most widespread, using mainly branched-chain and aromatic amino acids, such as tryptophan (Fig. 5), as substrates. However, amino acids with small nonbranched side chains, such as alanine and glycine, are poor substrates for system L. It should also be noted that the substrate specificity of transporters in neoplastic cells may be different from the corresponding normal tissue (26,27).

In contrast to the studies of sodium dependency there was a discrepancy between the influence of chloride ions on 5-ALA-ME uptake and PpIX formation (Fig. 4). It was found that 5-ALA-ME uptake was independent of the extracellular chloride concentration while the rate of PpIX formation was reduced by more than a factor of two in medium without chloride. Although it is well known that parameters such as ionic strength and pH influence on activity of the heme pathway enzymes (28–30) we are not aware of any studies of the importance of chloride. The intracellular concentration of chloride is usually relatively high (5–15 mM) and a substantial reduction in the intracellular concentration may have direct or indirect effects on the heme pathway enzymes.

In conclusion, 5-ALA-ME is transported into WiDr cells in a Cl^- -independent manner, it is partly Na^+ dependent and 50–60% inhibited by nonpolar amino acids. The cellular uptake is reduced by low temperatures and completely inhibited by metabolic inhibitors. The results indicate that 5-ALA-ME is taken up by active transport mechanisms, and it is likely that more than one transporter is involved in the cellular uptake of 5-ALA-ME. About 50% of the 5-ALA-ME transport occurs on carriers for glycine, but different from System Gly.

Acknowledgements—We are grateful to Yorkshire Cancer Research

(UK) and to Program in Medical Technology, Faculty of Medicine, Norwegian University of Science and Technology (Trondheim, Norway) for financial support.

REFERENCES

- Peng, Q., T. Warloe, K. Berg, J. Moan, M. Kongshaug, K. E. Giercksky and J. M. Nesland (1997) 5-aminolevulinic acid-based photodynamic therapy—clinical research and future challenges. *Cancer* **79**, 2282–2308.
- Gaullier, J. M., K. Berg, Q. Peng, H. Anholt, P. K. Sélbo, L. W. Ma and J. Moan (1997) Use of 5-aminolevulinic acid esters to improve photodynamic therapy on cells in culture. *Cancer Res.* **57**, 1481–1486.
- Kloek, J. and G. M. J. B. Van Henegouwen (1996) Prodrugs of 5-aminolevulinic acid for photodynamic therapy. *Photochem. Photobiol.* **64**, 994–1000.
- Fritsch, C., J. Batz, K. Bolsen, K. W. Schulte, M. Zumdick, T. Ruzicka and G. Goerz (1997) *Ex vivo* application of δ -aminolevulinic acid induces high and specific porphyrin levels in human skin tumors: possible basis for selective photodynamic therapy. *Photochem. Photobiol.* **66**, 114–118.
- Gederaas, O. A., K. Berg and I. Romslo (2000) A comparative study of normal and reverse phase high pressure liquid chromatography for analysis of porphyrins accumulated after 5-aminolevulinic acid treatment of colon adenocarcinoma cells. *Cancer Lett.* **150**, 205–213.
- Uehlinger, P., M. Zellweger, G. Wagnieres, L. Juillerat-Jeanneret, H. van den Bergh and N. Lange (2000) 5-Aminolevulinic acid and its derivatives: physical chemical properties and protoporphyrin IX formation in cultured cells. *J. Photochem. Photobiol. B: Biol.* **54**, 72–80.
- Casas, A., A. M. D. Battle, A. R. Butler, D. Robertson, E. H. Brown, A. MacRobert and P. A. Riley (1999) Comparative effect of ALA derivatives on protoporphyrin IX production in human and rat skin organ cultures. *Br. J. Cancer* **80**, 1525–1532.
- Berger, Y., A. Greppi, O. Siri, L. Juillerat and R. Neier (1999) New 5-aminolevulinic acid derivatives for selective protoporphyrin IX production in cells. 2nd Internet Conference of *Photochemistry and Photobiology*. <http://www.photobiology.com/photobiology99/contrib/neier/index.htm>
- Peng, Q., J. Moan, T. Warloe, V. Iani, H. B. Steen, A. Bjørseth and J. M. Nesland (1996) Build-up of esterified aminolevulinic acid-derivative-induced porphyrin fluorescence in normal mouse skin. *J. Photochem. Photobiol. B: Biol.* **34**, 95–96.
- Fritsch, C., B. Homey, W. Stahl, P. Lehmann, T. Ruzicka and H. Sies (1998) Preferential relative porphyrin enrichment in solar keratoses upon topical application of δ -aminolevulinic acid methylester. *Photochem. Photobiol.* **68**, 218–221.
- Sørensen, R., P. Juzenas, V. Iani and J. Moan (2000) Formation of protoporphyrin IX in mouse skin after topical application of 5-aminolevulinic acid and its methyl ester. *Photochemother. Cancer Other Dis.* **77**–81.
- Peng, Q., K. Berg, J. Moan, M. Kongshaug and J. M. Nesland (1997) 5-aminolevulinic acid-based photodynamic therapy: principles and experimental research. *Photochem. Photobiol.* **65**, 235–251.
- Rud, E. and K. Berg (1998) Characterisation of the cellular uptake of 5-aminolevulinic acid. *Photochemotherapy: Photodyn. Ther. Other Modalities IV* **3563**, 28–37.
- Rud, E., O. A. Gederaas, A. Høgset and K. Berg (2000) 5-Aminolevulinic acid, but not 5-aminolevulinic acid esters, is transported into adenocarcinoma cells by system beta transporters. *Photochem. Photobiol.* **71**, 640–647.
- Noguchi, P., R. Wallace, J. Johnson, E. M. Earley, S. O'Brien, S. Ferrone, M. A. Pellegrino, J. Milstien, C. Ncedy, W. Browne and J. Petricciani (1979) Characterization of the WIDR: a human colon carcinoma cell line. *In Vitro* **15**, 401–408.
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Darzynkiewicz, Z., X. Li and J. Gong (1994) Assays of cell viability: discrimination of cells dying by apoptosis. *Methods Cell Biol.* **41**, 15–38.
- Miyamoto, Y., H. Nakamura, T. Hoshi, V. Ganapathy and F. H. Leibach (1990) Uphill transport of beta-alanine in intestinal brush-border membrane vesicles. *Am. J. Physiol.* **259**, G372–G379.
- Bermudez Moretti, M., S. R. Correa Garcia, M. S. Chianelli, E. H. Ramos, J. R. Mattoon and A. Battle (1995) Evidence that 4-aminobutyric acid and 5-aminolevulinic acid share a common transport system into *Saccharomyces cerevisiae*. *Int. J. Biochem. Cell Biol.* **27**, 169–173.
- Palacin, M., R. Estevez, J. Bertran and A. Zorzano (1998) Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol. Rev.* **78**, 969–1054.
- Klock, J., W. Akkermans and G. M. J. B. Van Henegouwen (1998) Derivatives of 5-aminolevulinic acid for photodynamic therapy: enzymatic conversion into protoporphyrin. *Photochem. Photobiol.* **67**, 150–154.
- Zafra, F., C. Aragon and C. Gimenez (1997) Molecular biology of glycinergic neurotransmission. *Mol. Neurobiol.* **14**, 117–142.
- Christensen, H. N., D. L. Oxender, M. Liang and K. A. Vatz (1965) The use of N-methylation to direct route of mediated transport of amino acids. *J. Biol. Chem.* **240**, 3609–3616.
- Christensen, H. N., M. Liang and E. G. Archer (1967) A distinct Na⁺-requiring transport system for alanine, serine, cysteine, and similar amino acids. *J. Biol. Chem.* **242**, 5237–5246.
- Lynch, A. M. and J. D. McGivan (1987) Evidence for a single common Na⁺-dependent transport system for alanine, glutamine, leucine and phenylalanine in brush-border membrane vesicles from bovine kidney. *Biochim. Biophys. Acta* **899**, 176–184.
- McGivan, J. D. (1998) Rat hepatoma cells express novel transport systems for glutamine and glutamate in addition to those present in normal rat hepatocytes. *Biochem. J.* **330**, 255–260.
- Dudeck, K. L., E. E. Dudenhausen, T. C. Chiles, P. Fafournoux and M. S. Kilberg (1987) Evidence for inherent differences in the system A carrier from normal and transformed liver tissue. Differential inactivation and substrate protection in membrane vesicles and reconstituted proteoliposomes. *J. Biol. Chem.* **262**, 12565–12569.
- Poulson, R. and W. J. Polglase (1975) The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX. Protoporphyrinogen oxidase activity in mitochondrial extracts of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **250**, 1269–1274.
- Sorianello, E. M. and M. B. Mazzetti (2000) Function and structure of rat hepatic coproporphyrinogen oxidase. *Comp. Biochem. Physiol. B.* **127**, 155–164.
- Straka, J. G. and J. P. Kushner (1983) Purification and characterization of bovine hepatic uroporphyrinogen decarboxylase. *Biochemistry* **22**, 4664–4672.